BULG. J. PLANT PHYSIOL., 1995, 21(2–3), 80–97

PARADOXICAL RESULTS IN THE ANALYSIS OF HYPERHYDRIC TISSUES CONSIDERED AS BEING UNDER STRESS: QUESTIONS FOR A DEBATE

Thomas Gaspar[1], Claire Kevers[1], Thierry Franck[1], Badia Bisbis[1], Jean-Pierre Billard[2], Claude Huault[2], Frédérik Le Dily[2], Geneviève Petit-Paly[3], Marc Rideau[3], Claude Penel[4], Michèle Crèvecoeur[4], Hubert Greppin[4]

[1]Hormonologie Fondamentale et Appliquée, Institut de Botanique B 22, Université - Sart Tilman, B-4000 Liège, Belgium
[2]Laboratoire de Physiologie et Biochimie Végétales, Université de Caen, F-14032 Caen, France
[3]Laboratoire de Biologie Cellulaire et Biochimie Végétale, Université de Tours, avenue Monge, 31, F - 37200 Tours, France
[4]Laboratoire de Physiologie Végétale, Université de Genève, place de l'Université 3, CH-1211 Genève 4, Switzerland

Summary. Hyperhydric malformations affecting shoots under micropropagation, a phenomenon called vitrification or hyperhydricity, appear as resulting from an unability of the organs to adapt completely their whole defense battery composed of enzymes against activated oxygen species and of soluble reductants, in front of several simultaneous stresses due to *in vitro* culture conditions. Two different types of calli, habituated to both auxins and cytokinins, show typical characteristics of hyperhydricity. Although they appear to be protected against deleterious reactions generated by stresses, behaviour in darkness instead of light and several biochemical features prone for the calli still being tissues under permanent stress. Paradoxical results unexplained by literature data lead to a reappraisal of fundamental questions related to stresses, especially in relation to hyperhydricity and habituation.

Key words: defense enzymes, habituation, hyperhydricity, plant tissue cultures, stress

Hyperhydric malformations as a response to stress conditions

The term vitrification, recently rebaptized hyperhydricity (Debergh et al., 1992), encompasses a teratological process occurring during the *in vitro* multiplication of plants (reviews by Debergh et al., 1981; Kevers et al., 1984; Gaspar et al., 1987; Pâques and Boxus, 1987; Ziv, 1991; Gaspar, 1991). This physiological disorder is apparent through so-called hyperhydric malformations affecting stems and leaves. The malformations (stems thickened with shorter internodes, thick leaves frequently elongated, wrinkled and/or curled, and brittle) result from:

- deficiencies of cell wall edification, namely cellulose and lignin deposition (Kevers and Gaspar, 1985a; Kevers et al., 1987, 1988) and associated deficiency in organisation of certain tissues such as the vascular bundles (Leshem, 1983a; Ziv et al., 1983) and the palisade tissue (Brainerd et al., 1981; Debergh et al., 1981; Vieitez et al., 1987);
- deficiencies in cuticule and wax deposition (Grout and Aston, 1978; Sutter and Langhans, 1979; Ziv et al., 1983, 1987; Aitken-Christie et al., 1985; John, 1986; Dhawan and Bhojwani, 1987) and of stomata closure (Brainerd et al., 1981; Leshem, 1983a,b; John, 1986);
- reduced cell-to-cell adhesion resulting in breakability of the organs;
- deficiency of chlorophyll accumulation (Hegedus and Phan, 1983; Phan and Letouzé, 1983; Ziv et al., 1983; Franck et al., 1995);
- hyperhydricity resulting in watery tissues (Kevers et al., 1984; Crèvecoeur et al., 1987). The water in excess has been shown to be merely located in the intercellular spaces and in the foamy walls (Kevers and Gaspar, 1986).

These symptoms appear at different degrees. At extremes, vitrification leads to disfunctioning of the primary meristems (the appearance of fasciated stems is the first most clearcut visible manifestation) and even to their death through the well known phenomenon of (humid) apex necrosis (Kataeva et al., 1991; Gaspar et al., 1991).

Hyperhydricity affects shoots at the proliferation stage, never at the rooting stage (Gaspar et al., 1991). The above structural teratomas have been associated with several biochemical deviations concerning nitrogen metabolism (Letouzé and Daguin, 1983; Daguin and Letouzé, 1985, 1986), the synthesis of tetrapyrrole-containing compounds including chlorophylls (Franck et al., 1995), the phenolic metabolism (Hedegus and Phan, 1983; Kevers et al., 1984), the activity of the different peroxidase types (Kevers et al., 1984; Kevers and Gaspar, 1985a; Letouzé and Daguin, 1987), in relation with auxin and ethylene metabolism (Kevers et al., 1984; Kevers and Gaspar, 1985b; Gaspar, 1986; Gaspar et al., 1987; Phan, 1991) and lignification (Daguin and Letouzé, 1985; Kevers and Gaspar, 1985a; Kevers et al., 1987; Letouzé et Daguin, 1987). A tentative pathway of the biochemical events leading to vitrification has been

proposed by Kevers et al. (1984). This pathway was considered as an adaptive response of the tissues submitted to several stresses simultaneously. Such stresses can be listed as follows:

- injury due to the prior dissection of the explants;
- the osmotic shock caused by infiltration of the culture medium into the intercellular spaces (Böttcher and Göring, 1987; Böttcher et al., 1988) confirmed by typical fluxes of Ca and K ions (Kevers and Gaspar, 1986);
- the high relative humidity of the flask atmosphere, merely in liquid or floppy soft media (Debergh and Maene, 1984; Williams and Taji, 1991);
- the high ammonium content of the media (Daguin and Letouzé, 1986);
- the confined atmospheres of the jars accumulating ethylene and other gases (Kevers and Gaspar, 1985b; Dillen and Buysens, 1989; Phan, 1991; Demeester et al., 1995);
- the high amount of cytokinins (benzyladenine more than other cytokinins is efficient) or of substituted ureas (thidiazuron for instance) whose penetration in the tissue is facilitated on soft media (Debergh, 1983; Bornman and Vogelmann, 1984; Leshem et al., 1988; Williams and Taji, 1991; Cambecèdes et al., 1991).

In this context, several authors have observed that isopentenyl transferase transformed-shoot cultures of tobacco (which overproduce cytokinins) have fasciated stems with thick and breakable leaves, and often accumulate decreased amounts of chlorophyll (Memelink et al., 1987; Smigocki et al., 1988; Hamdi et al., 1995). Therefore, these transgenic shoots resemble vitrified shoots. There is also evidence that both exogenously applied- and endogenously produced-cytokinins give responses similar to those achieved by various stresses (Harding and Smigocki, 1991). Moreover it was proposed that cytokinin act as a signal transducer coordinating metabolic changes in *Mesembryanthemun cristallinum* leaves with the water status of whole plant (Thomas et al., 1992). Changes in water contents are clearly an important feature of vitrified tissues.

Cell wall and plasma membrane synergistically produce some NADH oxidation activities capable of generating superoxide anion and hydrogen peroxide (Fig. 1) playing several physiological roles namely in defense mechanisms (Vianello and Macri, 1991). Membrane systems however are the primary sites of stresses action. Stresses, whatever their kinds, generate different activated forms of oxygen (Fig. 2) which can be deleterious for the tissues, causing damages at the membrane itself (Dhindsa et al., 1981; Thompson et al., 1987; Winston, 1990; Kumar and Knowles, 1993; Bladier et al., 1994). Free radical mediated-membrane disruption involves de-esterification of membrane phospholipids, accumulation of saturated free fatty acids, and peroxidations forming deleterious aldehydes (Arbillot et al., 1991) (Fig. 3). Plant tissues as animal tissues however are normally equipped of a battery of defence systems against these toxic oxygen forms including reducing enzymes and reducing sub-

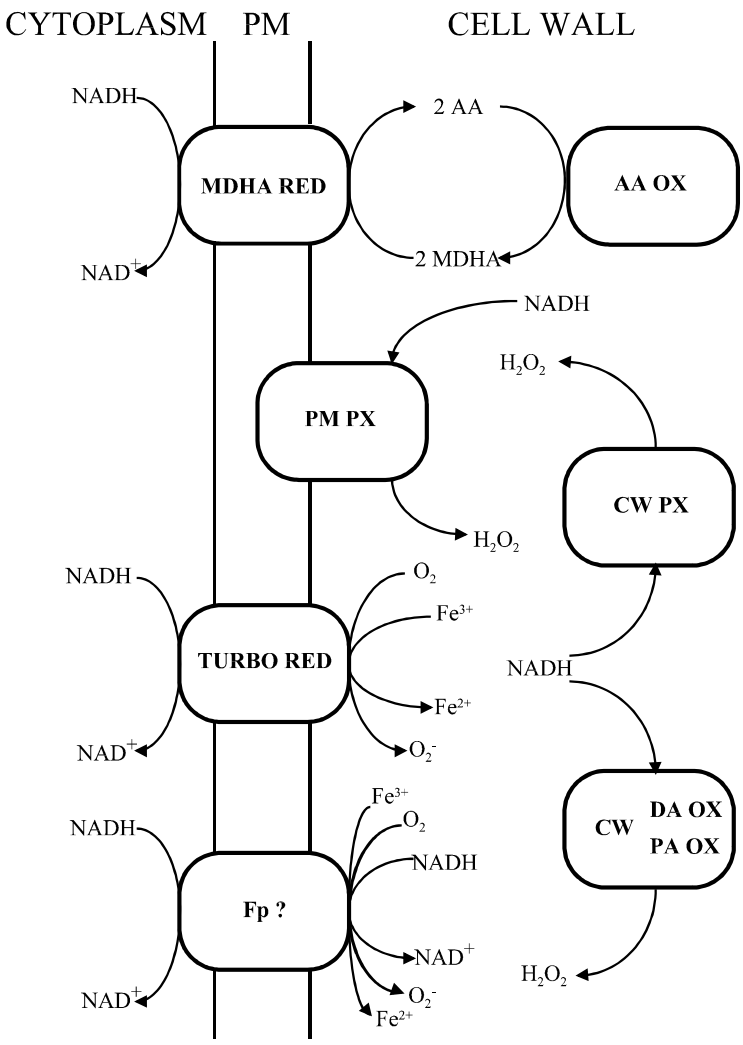

Fig. 1. Synergistic action of cell wall and plasma membrane in NADH oxidases mediated generation of free radicals and hydrogen peroxide at the surface of plant cells (AA = ascorbate; CW = cell wall; DA = diamine; Fp = flavoprotein; MDHA = monodehydroascorbate; OX = oxidase; PA = polyamine; PX = peroxidase; RED = reductase; TURBO = transmembrane redox system), (adapted from Vianello and Macri, 1991)

stances listed in Fig. 2. Considering vitrifying and vitrified tissues under stress conditions, we have attempted to evaluate their capacity to adapt their defence systems against activated forms of oxygen using several approaches and three different plant model materials. We came to be confronted with paradoxical results unexplained with the literature of our knowledge. We simply want to open the debate hoping that it will enrich the experimental approach of the process of hyperhydricity.

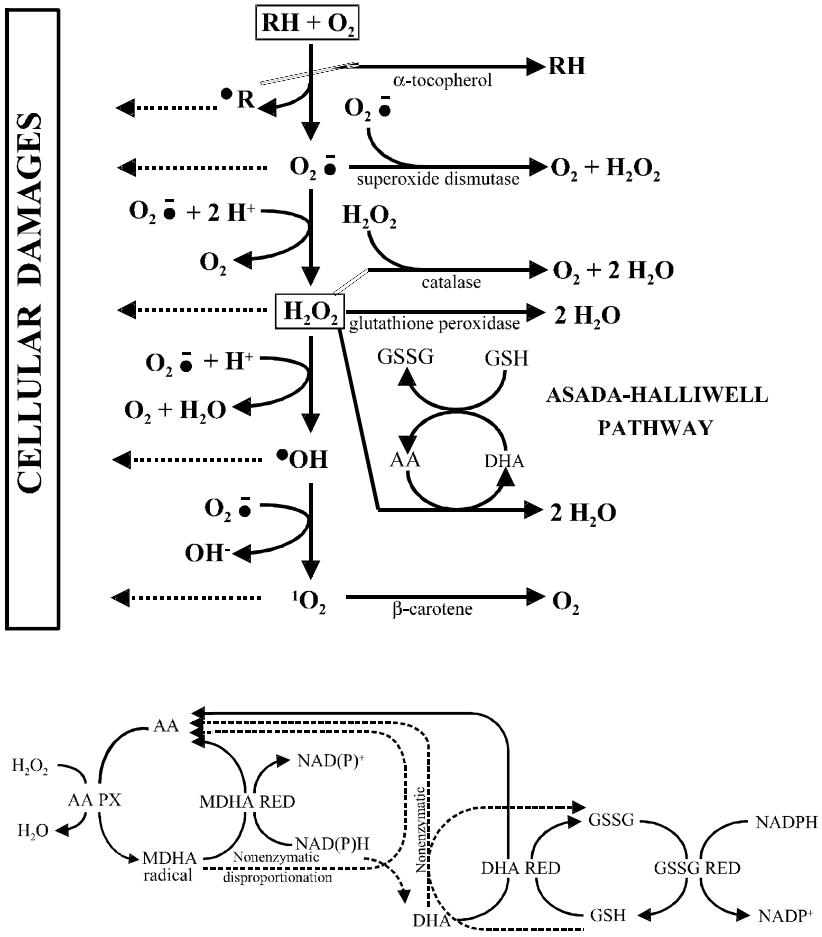

Fig. 2. Free-radical chain reaction forming activated oxygen forms leading to cellular damages, and the possible defense mechanisms (enzymes and reductants). The Asada–Halliwell pathway is detailed. (Additional abbreviations from Fig. 1: DHA = dehydroascorbate; GSH = reduced glutathione; GSSG = oxidized glutathione)

Vitrification of shoots of *Prunus avium* and its reactions

Normal shoots of *Prunus avium* are produced through axillary proliferation using benzyladenine (1 mg.l^{-1}) as cytokinin in a medium solidified by agar (8 g.l^{-1}) in four weeks multiplication cycles. The substitution of agar by gelrite (2.5 g.l^{-1}), allowing the medium to become somehow softer, induces vitrification of 100% of the shoots within three weeks, the first morphological symptoms becoming clearly visible from the 7th day (Franck et al., 1995). Such a vitrifying effect of gelrite had been shown before by Zimmerman et al. (1991) and Bonga and Von Aderkas (1992). By com-

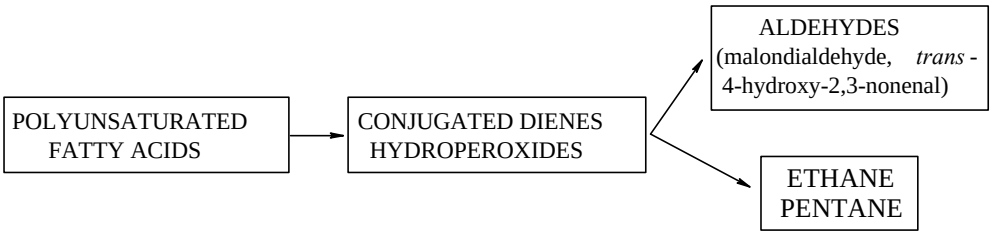

Fig. 3. Degradation products resulting from the oxidation of polyunsaturated fatty acids

parison with normal shoots produced on agar, the vitrifying shoots accumulate less chlorophylls. As shown from the results of Table 1, vitrifying shoots (on gelrite), com-

Table 1. Relative activities or amounts of stress defense systems and markers in fully habituated nonorganogenic (HNO) calli (vs normal (N) ones) and in vitrified (V) shoots (vs normal (N) ones), (↑ means higher; ↓ means lower)

	Sugarbeet callus HNO/ N (after 14 days of culture)	**Wild sherry shoots** V/N (after 28 days of culture)
Enzymatic systems of defence		
SOD	↑	=
CAT	↓	↓
G PX	↓	↓
AA PX	↑	↓
MDHA RED	↓	↓
DHA RED	↑	↓
G RED	↑	↓
Water soluble reductants		
Ascorbate	/	↓
Dehydroascorbate	/	↓
Glutathione	↓	=
Exogenous reducing power		
Ferricyanide reduction	↓	↓
Markers of lipid peroxidation		
Peroxide index	↓	↓
MDA	↑	↓
Other markers of stress		
Proline	↑	↑
PAs	↑	↑

pared with normal shoots (on agar), develop a higher superoxide dismutase (SOD) activity, as do generally most of the plants submitted to physical or chemical stresses. SOD disembarrasses the tissue of the accumulated superoxide ions by transforming them into H_2O_2. In most plants under stress, this H_2O_2 generally is itself eliminated through catalase and through a reinforcement of the so-called Halliwell–Asada pathway (Foyer and Halliwell, 1976; Asada and Takahashi, 1987; Jahnke, 1991) involving ascorbate peroxidase, mono- and dehydro-ascorbate reductases and glutathione reductase, and ascorbate and glutathione as substrates (Fig. 2). These defence enzymes have been determined with lower activities in the vitrifying shoots of *Prunus avium* which has led to the conclusion that the hyperhydric malformations resulted from the inability of the shoots, submitted to different stresses simultaneously, to adapt normally their battery of defence enzymes against the toxic oxygen forms (Franck et al., 1995). Exactly the same conclusion has been reached quite independently by Sankhla (see comments in Agricell Reports vol. 23, number 2). The further analysis of free ascorbate and dehydroascorbate levels in the same vitrifying material indicated levels lower than in normal shoots (Franck et al.,1996) apparently in agreement with the above hypothesis. The hyperhydric malformations including necroses (limited at leaf borders at this stage) thus might well result of nonprevented deterioration at the levels of membranes. Malondialdehyde (MDA) classically is considered as an indicator of deleterious peroxidation at the membrane level (Kosugi and Kirugawa, 1989). It is measured as the major aldehyde reacting with thiobarbituric acid (TBA) (Hagège et al., 1990b). TBA-reactive substances just have been determined in vitrifying *Prunus avium* shoots at levels lower than in normal shoots (Franck et al.,1996). The level of hydroperoxide which generally account for cell biomembrane damages (Winston, 1990; Deby, 1991) was also determined with lower levels in vitrifying shoots. These two results are apparently in contradiction with the above hypothesis of unadaptation to stress.

A fully habituated callus of sugarbeet as a model of hyperhydric tissue

A fully habituated (whose growth became independent of exogenous application of auxin and cytokinin) line of sugarbeet in our hands since 1981 (Kevers et al., 1981) has been described with several typical characteristics of a hyperhydric tissue:

- hyperhydricity and watery aspect (Gaspar et al., 1988);
- deficiency of cell wall structuration (Crèvecoeur et al., 1987, 1992) with deficient lignification (Crèvecoeur et al., 1987; Hagège et al., 1991a);
- low levels of chlorophylls (Crèvecoeur et al., 1987; Bisbis et al., 1994);
- disturbed nitrogen metabolism (Le Dily et al., 1993a,c);

– friability (comparable to breakability in vitrified shoots), which has been attributed to a high degree of pectin esterification (Liners et al., 1994);
– susceptibility to necrosis when not subcultured through short (14 to 21 days) cycles (Hagège et al., 1992a; Le Dily et al., 1993b), which may correspond to the process of apex necrosis in vitrifying shoots;
– the loss of organogenic potential which may correspond or be a more advanced form of the diminished capacity of primary meristems of vitrified shoots to organize well structured stems and leaves (Bornmann and Vogelman, 1984; Gaspar et al., 1991).

Is the fully habituated sugarbeet callus under stress ?

Several biochemical features of this callus prone for it being a tissue under permanent stress (Le Dily et al. 1993b):

– first, SOD, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, and ascorbate peroxidase were found to have higher activities in the habituated (H) callus compared to a normal (N) one (Kevers et al., 1992; Hagège et al., 1992a).
– second, the H callus contains high levels of polyamines (Kevers et al., 1985; Hagège et al., 1990a; Le Dily et al., 1991) which generally accumulate in response to environment stresses (Slocum and Weinstein, 1990). Polyamines are considered as having scavenging properties for free radicals (Drolet et al., 1986; Mahmoud and Melo, 1991). The peroxide index of the H callus is very low (Hagège et al., 1990a) which is in agreement with its strong scavenging capacities. An experimentation comparing chemiluminescence from N and H cells under the effect of SOD and catalase indicated that H cells produced less chemiluminescent species and less hydrogen peroxide than N cells (Carrié et al., 1994), again in agreement with above results showing that H cells had a better protection against oxygen free radicals than N cells.
– thirdly, the H callus behaves as an ammonia-stressed tissue through altered nitrogen metabolism, as compared with a N callus. The H callus has higher NH_4^+ and glutamate contents that the N callus. It is well known that stress alters ammonia metabolism (Lovatt, 1990). In these ammonia conditions the mitochondrial glutamate deshydrogenase (GDH) may function in the direction of the glutamate synthesis (Yamaka and Oak, 1987). There is indeed an increase of GDH activity in the H callus (Le Dily et al., 1993a). It may be the result of *de novo* synthesis in response to ammonia as demonstrated in various stress conditions (Srivastava and Singh, 1987). This result is corroborated by the findings of

Letouzé and Daguin (1983) who demonstrated the appearance of a GDH isoenzyme in vitrified cherry cuttings obtained on excessive NH_4^+ media. The elevated level of GDH would explain the higher production of glutamate in the H callus. This callus is porphyrin-deficient as most of the vitrified tissues (see above). The lack of chlorophylls which are built from glutamate (Beale and Weinstein, 1989) thus may contribute to the high glutamate content

– fourthly, proline, which is a good indicator of stress conditions (Bellinger and Lahrer, 1987), accumulates at a higher level in the H callus and it has been shown that proline accumulating in the H callus was the major source of ornithine for polyamines synthesis (Le Dily et al., 1993d).

An additional photooxidative stress might be superposed. Indeed the transfer of the albino habituated sugarbeet callus to darkness improved its growth and delayed necrosis without affecting the nitrogen compounds such as glutamate, proline and polyamines (Kevers et al., 1995). The favourable effect of darkness might be attributed to the recovery of carotenoids (Bisbis et al., 1994; Kevers et al., 1995). The parallel recovery of peroxidase activity might also be questioned. Darkness reduced the level of TBA-reactive substances in the habituated callus while the reverse was observed in the normal green one.

Is the fully habituated periwinkle cell line C20A under stress?

A fully habituated line of periwinkle (*Catharanthus roseus*) called C20A has been selected from the 2,4-D-dependent line C20 ten years ago (Mérillon et al., 1986). Some characteristics of this line have been described (Mérillon et al., 1989; Ouelhazi et al., 1993; Mérillon et al., 1994, 1995a, b). The line has not been investigated as thoroughly as has been the habituated sugarbeet line in terms of stress reactions but the following conclusions may be drawn:

– first, habituation changes the lipid composition of microsomal membranes and is associated with a rise in membrane fluidity (Mérillon et al., 1995a). As already observed by Arbillot et al. (1991) in senescing habituated calli of sugarbeet, the 18:1 to 18:2 ratio is increased but in contrast with results reported by these authors, comparable malondialdehyde contents are observed in both the 2,4-D-dependent line and the habituated line. It is known that an increase in membrane fluidity is often accompanied by a decrease of desaturase activities (Brown et al., 1987 and references therein): this may explain the decrease in the 18:2 relative proportion in C20 cells;

– second, preliminary investigations on activities of some enzymes associated with defence mechanisms against activated oxygen forms, show that catalase, ascorbate peroxidase and glutathione-reductase are increased in the line C20A as com-

pared to line C20. Moreover, putrescine contents are about 5-fold higher in the C20A line than in the C20 line. These data suggest that the fully habituated line has stronger scavenging capacities than the 2,4-D-dependent line.

Paradoxical results and questions

Vitrifying shoots and sugarbeet habituated calli both are hyperhydric materials and both appear under continuous stress. This may be related to their unability to overcome several simultaneous stresses by adapting completely their battery of defence mechanisms against activated oxygen forms. This uncapacity was different. Both tissues increased their SOD activity but "failed" to adapt catalase activity. Vitrifying shoots also failed to adapt the Asada–Halliwell system involving ascorbate peroxidase and glutathione reductase and their water-soluble reductants. The habituated callus had higher activities of the enzymes of the Asada–Halliwell system but had lower or no content of ascorbate, dehydroascorbate and glutathione levels than the normal one. The latter result might be interpreted as an unadaptation to stress due to the absence of reductors or on the contrary as a good adaptation, the low level of reductants being due to a high turn-over of the Asada–Halliwell pathway. The peroxide index is relatively low indeed in the habituated callus and crude extracts show an anti-lipoperoxidant potential higher than this of the N callus (Hagège et al., 1993), but the MDA level is high which is another paradox. Also paradoxical was that vitrifying shoots lowered both their peroxide index and their MDA level (Franck et al., 1996). The formal acquaintance between the peroxide index and the MDA level, and their relationships with stress, therefore have to be reappraised. Can we imagine that MDA may originate from other sources than membrane lipids? Do we miss some of the *in vivo* realities due to analyses in somehow crude extracts *in vitro*? Crude extracts of the sugarbeet habituated callus indeed exhibited poor peroxidase activity (Kevers et al., 1981; Gaspar et al., 1988; Hagège et al, 1992b) while purified plasmamembranes exhibited high activity (Hagège et al., 1991b). In such teratomas, it may also happen that different compartments from the abnormally structurated cells react in opposite manners.

As far as the differences between vitrifying wild cherry shoots and sugarbeet habituated calli are concerned, of course it must be considered that the formers enter a neoplasic progression while the latters are at the end, finishing with true cancerous cells (Gaspar et al., 1991). In this context, the strong antioxidant properties of the fully habituated cell line might be correlated with its higher level of auxin protectors (Kevers et al., 1981; Gaspar et al., 1988). Stonier (1970) and Stonier and Yang (1971, 1973) indeed already established a correlation between deficient differentiation of plant tumor cells and auxin protector content. As already proposed (Le Dily et al., 1993a), the habituated cells might result from punctual mutations occurred

while they tended to protect themselves against the deleterious radicals under the stresses, when they entered *in vitro*. This adaptation might have resulted in the increase in the free radical scavenging properties through polyamines and to a decrease of the functioning of the free radical-producing systems (photosynthesis? auxin catabolism ? ethylene formation ?). Cells from the habituated callus finally appear as cells having succeeded partially in escaping culture stressing conditions: either they still cannot avoid some of the stresses (see above) or their modified structures have rendered them still more susceptible to some of them. Such questions and such a debate have also been raised for the immortalization and malignant transformation of animal cells (Oberley and Oberley, 1988).

References

Aitken-Christie, J., C. Jones, S. Bond, 1985. Wet and waxy shoots in *radiata* pine micropropagation. Acta Hortic., 166, 93-100.

Arbillot, J., J. Le Saos, J.P. Billard, J. Boucaud, Th. Gaspar, 1991. Changes in fatty acid and lipid composition in normal and habituated sugarbeet calli. Phytochem., 30, 491-494.

Asada, K., M. Takahashi, 1987. Production and scavenging of active oxygen in photosynthesis. In: Photoinhibition. Eds. D.J. Kyle, C.B. Osmond, C.J. Arntzen, Elsevier Science Publ., 227-287.

Beale, S.I., J.D. Weinstein, 1989. Tetrapyrrole metabolism in photosynthetic organisms. In: Heme and Chlorophyll Biosynthesis. Ed. H.A. Daily, McGraw Hill, New York, 287-291.

Bellinger, Y., F. Lahrer, 1987. Proline accumulation in higher plants: a redox buffer. Life Sci. Adv., 6, 23-27.

Bisbis, B., E. Dujardin, C. Kevers, D. Hagège, Th. Gaspar, 1994. Chlorophylls and carotenoids in a fully habituated nonorganogenic callus of *Beta vulgaris*. Biol. Plant., 36, 443-449.

Bladier, C., P. Carrier, P. Chagvardieff, 1994. Light stress and oxidative cell damage in photoautotrophic cell suspension of *Euphorbia characias* L. Plant Physiol., 106, 941-947.

Böttcher, I., H. Göring, 1987. Die Vitrifikation der Pflantzen bei der *in vitro* Kultur als Infiltration Problem. Biol. Rundsch., 25, 191-193.

Böttcher, I., K. Zoglauer, H. Göring, 1988. Induction and reversion of vitrification of plants cultured *in vitro.* Physiol. Plant., 72, 560-564.

Bonga, J.M.,P.V. Aderkas, 1992. *In vitro* Culture of Trees. Kluwer Acad. Publ., Dordrecht.

Bornman, C.H., T.C. Vogelmann, 1984. Effect of rigidity of gel medium on benzyladenine-induced adventitious bud formation and vitrification *in vitro Picea abies*. Physiol. Plant., 61, 505-512.

Brainerd, K.E., L.H. Fuchigami, S. Kwiatkowski, C.S. Clark, 1981. Leaf anatomy and water stress of aseptically cultured Pixy plum grown under different environments. Hort Sci. 16, 173-175.

Brown, J.H., D.V. Lynch, J.E. Thompson, 1987. Molecular species specificity of phospholipid breakdown in microsomal membranes of senescing carnation flowers. Plant Physiol., 85, 679-683.

Cambecèdes, J., M. Duron, L. Decourtye, 1991. Adventitious bud regeneration from leaf explants of the shrubby ornamental honeysuckle, *Lonicera nitida* Wils. cv. "Maigrün": effects of thidiazuron and 2,3,5- triidobenzoic acid. Plant Cell Reports, 10, 471-474.

Carrié, B., Th. Gaspar, H. Greppin, C. Penel, 1994. Redox characteristics of normal and habituated cell lines of sugarbeet. Plant Cell Environment, 17, 457-461.

Crèvecoeur, M., C. Kevers, Th. Gaspar, 1987. A comparative biochemical and cytological characterization of normal and habituated sugarbeet calli. Biol. Plant., 29, 1-6.

Crèvecoeur, M., D. Hagège, A.M. Catesson, H. Greppin, Th.Gaspar, 1992. Ultrastructural characteristics of cells from normal and habituated sugar beet calli. Plant Physiol. Biochem., 30, 87-95.

Daguin, F., R. Letouzé, 1985. Relations entre hypolignification et état vitreux chez S*alix babylonica* en culture *in vitro*. Rôle de la nutrition ammoniacale. Can. J. Bot., 63, 324-326.

Daguin, F., R. Letouzé, 1986. Ammonium-induced vitrification in cultured tissue. Physiol. Plant., 66, 94-98.

Debergh, P.C., 1983. Effect of agar brand and concentration on the tissue culture medium. Physiol. Plant., 59, 270-276.

Debergh, P., J. Aitken-Christie, D. Cohen, B. Grout, S. von Arnold, R. Zimmerman, M. Ziv, 1992. Reconsideration of the term *vitrification* as used in micropropagation. Plant Cell Tissue Organ Cult., 30, 165-140.

Debergh, P.C.,Y. Harbaoui, R. Lemeur, 1981. Mass propagation of globe artichoke (*Cynara scolymus*): evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant., 53, 181-187.

Debergh, P.C., L.J. Maene, 1984. Preparation of tissue cultured plants for rooting and establishment *in vivo*. In: Proc. Int. Symp. Plant. Tissue Cell. Cult. Applic. Crop Improv. Eds. F.J. Novak, T. Havel, K. Dolezel, Czechoslovakia Acad. Sci., Prague, 487-495.

Deby, C. 1991. La biochimie de l'oxygène. La Recherche, 22, 56-64.

Demeester, J.J., D.G. Matthÿs, B. Pascat, P.C. Debergh, 1995. Toward a controllable headspace composition. Growth, development, and headspace of a micropropagated *Prunus* rootstock in different containers. In Vitro Cell. Dev. Biol., 31, 105-112.

Dhawan, V., S.S. Bhojwani, 1987. Hardening *in vitro* and morpho-physiological changes in the leaves during acclimatization of micropropagated plants of *Leucaena*

leucocephala (Lam.) de Wit. Plant Science, 53, 65-72.

Dhindsa, R.J., P. Plumb-Dhindsa, T.A. Thorpe, 1981. Leaf senescence: correlated with increased levels of membrane permeability, lipid peroxidation and decreased levels of superoxide dismutase and catalase. J. Exp. Bot., 32, 93-101.

Dillen, W., S. Buysens, 1989. A simple technique to overcome vitrification in *Gypsophila paniculata* L. Plant Cell Tissue Organ Cult., 19, 181-188.

Drolet, G., E.B. Dumbroff, R.L. Legge, J.E. Thompson. 1986. Radical scavenging properties of polyamines. Phytochemistry, 25, 367-371.

Foyer, C.H., B. Halliwell, 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta, 133, 21-25.

Franck, Th., C. Kevers, Th. Gaspar, 1995. Protective enzymatic systems against activated oxygen species compared in normal and vitrified shoots of *Prunus avium* L. raised *in vitro*. Plant Growth Regul., 16,253-256.

Franck, T., C. Kevers, C. Penel, Th. Gaspar, H. Greppin, 1996. Changes in the level of water-soluble reductants, exogenous redox property, and markers of lipid peroxidation in normal and hyperhydrating shoots of *Prunus avium* L.. Plant Cell, Tissue Organ Cult. In press.

Gaspar, Th., 1986. Integrated relationships of biochemical and physiological peroxidase activities. In: Molecular and Physiological Aspects of Plant Peroxidases. Eds. H. Greppin, C. Penel, Th. Gaspar, Univ. Geneva, Switzerland, 455-468.

Gaspar, Th., 1991. Vitrification in micropropagation. In: Biotechnology in Agriculture and Forestry. Vol.17. High-Tech and Micropropagation I. Ed. Y.P.S. Bajaj, Springer-Verlag, Berlin, 117-126.

Gaspar, Th., D. Hagège, C. Kevers, C. Penel, M. Crèvecoeur, I. Engelmann, H. Greppin, J.M. Foidart, 1991. When plant teratomas turn into cancers in the absence of pathogens. Physiol. Plant., 83, 696-701.

Gaspar, Th., C. Kevers, P. Debergh, L. Maene, M. Pâques, Ph. Boxus, 1987. Vitrification : morphological, physiological and ecological aspects. In: Cell and Tissue Culture in Forestry. Vol. 1. General Principles and Biotechnology. Eds. J.M. Bonga, D.J. Durzan, Martinus Nijhoff, Dordrecht, 152-166.

Gaspar, Th., C. Kevers, C. Penel, M. Crèvecoeur, H. Greppin, 1988. Biochemical characterization of normal and habituated sugarbeet calli. Relationship with anatomy, habituation and organogenesis. Postdamer Forschungen B57, 20-30.

Grout, B.W.W., M.J. Aston, 1978. Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. Ann. Bot., 42, 993-995.

Hagège, D., C. Deby, C. Kevers, J.M. Foidart, Th. Gaspar, 1993. Anti-lipoperoxidant potential of the fully habituated nonorganogenic sugarbeet callus. Arch. Intern. Physiol. Biochim. Biophys., 101, PP9.

Hagège, D., C. Kevers, J. Boucaud, M. Duyme, Th. Gaspar, 1990a. Polyamines, phospholopids, and peroxides in normal and habituated sugar beet calli. J. Plant

Physiol., 136, 641-645.

Hagège, D.,C. Kevers, M. Crèvecoeur, M.T. Tollier, B. Monties, Th. Gaspar, 1991. Peroxidases, growth and differentiation of habituated sugarbeet cells. In: Biochemical, Molecular, and Physiological aspects of Plant Peroxidases. Eds. J. Lobarzewski, H. Greppin, C. Penel, Th. Gaspar, University of Lublin and University of Geneva, 281-290.

Hagège, D., C. Kevers, Ph. Salabert, Th. Gaspar, 1992a. Protective systems against activated oxygen species compared in normal and fully habituated nonorganogenic sugarbeet calluses. In vitro Cell Dev. Biol., 28, 143-147.

Hagège, D., A. Nouvelot, J. Boucaud, Th. Gaspar, 1990. Malondialdehyde titration with thiobarbiturate in plant extracts: avoidance of pigment interference. Phytochem. Anal., 1, 86-89.

Hagège, D.,C. Penel, M. Crèvecoeur, C. Kevers, Th. Gaspar, J. Boucaud, H. Greppin, 1991b. Plasmamembrane peroxidases from the auxin- and cytokinin-independent sugarbeet callus. Arch. intern. Physiol. Biochim. Biophys., 99, PP2.

Hagège, D., D. Werck-Reichhart, P. Schmitt, Th. Gaspar, 1992b. Deficiency in tetrapyrrole-containing compounds in a non-organogenic habituated sugar-beet cell line. Plant Physiol. Biochem., 30, 649-654.

Hamdi, S., J. Crèche, F. Garnier, M. Mars, A. Décendit, Th. Gaspar, M. Rideau, 1995. Cytokinin involvement in the control of coumarin accumulation in *Nicotiana tabacum*. Investigation with normal and transformed tissues carrying the isopentenyl transferase gene. Plant Physiol. Biochem., 33, 283-28.

Harding, S.A., A.C. Smigocki, 1994. Cytokinin modulates stress response genes in isopentenyl transferase-transformed *Nicotiana plumbaginifolia* plants. Physiol. Plant., 90, 327-333.

Hegedus, P., C.T. Phan, 1983. Actions de phénols sur les malformations observées chez les porte-greffes de pommiers M-26 et 0-3 cultivés *in vitro*. Rev. Can. Biol. Exp., 42, 33-38.

Jahnke, L.S., M.R. Hull, S.P. Long, 1991. Chilling stress and oxygen metabolizing enzyme in *Zea mays* and *Zea diploperennis*. Plant Cell Environment, 14, 97-104.

John, A., 1986. Vitrification in Sitka spruce culture. In: Plant Tissue Culture and its Agricultural Applications. Eds. L. Whithers, P.G. Alderson, Butterworth, London, 167-174.

Kataeva, N.V., I.G. Alexandrova, R.G. Butenko, E.V. Dragavtceva, 1991. Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured *in vitro*. Plant Cell Tissue Organ Cult., 27, 149-154.

Kevers, C., B. Bisbis, F. Le Dily, J.P. Billard, C. Huault, Th. Gaspar, 1995. Darkness improves growth and delays necrosis in a nonchlorophyllous habituated sugarbeet callus: biochemical changes. In Vitro Cell. Dev. Biol., 31, 122-126.

Kevers, C., M. Coumans, M.F. Coumans-Gilles, Th. Gaspar, 1984. Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. Physiol. Plant., 61, 69-74.

Kevers, C., M. Coumans, W. De Greef, M. Hofinger, Th. Gaspar, 1981. Habituation in sugarbeet callus : auxin content, auxin protectors, peroxidase pattern and inhibitors. Physiol. Plant., 51, 281-286.

Kevers, C., Th. Gaspar, 1985a. Soluble, membrane and cell wall peroxidase, phenylalanine ammonialyase, and lignin changes in relation to vitrification of carnation tissues cultured *in vitro*. J. Plant Physiol., 118, 41-48.

Kevers, C.,Th. Gaspar, 1985b. Vitrification of carnation *in vitro*: changes in ethylene production, ACC level and capacity to convert ACC to ethylene. Plant Cell Tissue Organ Cult., 4, 215-223.

Kevers, C.,Th. Gaspar, 1986. Vitrification of carnation *in vitro*: changes in water content, extracellular space, air volume, and ions levels. Physiol. Vég., 24, 647-653.

Kevers, C., Th. Gaspar, M. Crèvecoeur, 1985. Nature et teneur des polyamines de cals normaux et habitués de betterave sucrière. Effet des régulateurs de croissance du milieu de culture. Botan. Helvetica, 95, 117-120.

Kevers, C., R. Goldberg, J. Chu-Ba, Th. Gaspar, 1988. Composition of the walls of stem and leaves of vitrifying carnation. Biol. Plant., 30, 219-223.

Kevers, C., D. Hagège, C. Penel, H. Greppin, Th. Gaspar, 1992. Intra- and extra-cellular ascorbate peroxidase and ascorbate oxidase activities in normal and fully habituated sugarbeet calli. Arch. Intern. Physiol. Biochim. Biophys. 100, B15.

Kevers, C., R. Prat, Th. Gaspar, 1987. Vitrification of carnation *in vitro*: changes in cell wall mechanical properties, cellulose and lignin content. Plant Growth Regul., 5, 59-66.

Kosugi, H., K. Kirugawa, 1989. Potential thiobarbituric acid-reactive substances in peroxidized lipids. In: Free Radical Biology and Medicine, Maxwell Pergamon Macmillan plc, vol. 7, 205-207.

Kumar, G.N.M., N.R. Knowles, 1993. Changes in lipid peroxidation and lipolytic and free-radical scavenging enzyme activities during aging and sprouting of potato (*Solanum tuberosum*) seed-tubers. Plant Physiol., 102, 115-124.

Le Dily, F., J.P. Billard, J. Boucaud, 1991. Polyamine levels in relation to growth and NaCl concentration in normal and habituated sugarbeet callus cultures. Plant Cell Environment, 14, 327-332.

Le Dily, F., J.P. Billard, Th. Gaspar, C. Huault, 1993a. Disturbed nitrogen metabolism associated with the hyperhydric status of fully habituated callus of sugar-beet. Physiol. Plant., 88, 129-134.

Le Dily, F., J.P. Billard, C. Huault, C. Kevers, Th. Gaspar, 1993b. Fully habituated sugarbeet callus: under permanent stress? In vitro Cell. Dev. Biol., 29P, 149-154.

Le Dily, F., C. Huault, Th. Gaspar, J.P. Billard, 1993c. Does altered nitrogen metabolism and H_2O_2 accumulation explain the vitrified status of the fully habituated callus of *Beta vulgaris* (L.). Plant Cell Tissue Organ Cult., 35, 69-74.

Le Dily, F., C. Huault, Th. Gaspar, J.P. Billard, 1993d. Gabaculine as a tool to investigate the polyamine biosynthesis pathway in habituated callus of *Beta vulgaris* (L.). Plant Growth Regul., 18, 221-223.

Leshem, B., 1983a. Growth of carnation meristems *in vitro*: anatomical structure of abnormal plantlets and the effect of agar concentration in the medium on their formation. Ann. Bot., 52, 413-415.

Leshem, B., 1983b. The carnation succulent plantlet, a stable teratological growth. Ann. Bot., 52, 873-876.

Leshem, B., D.P. Shaley, S. Izhar, 1988. Cytokinin as an inducer of vitrification in melon. Ann. Bot., 61, 255-260.

Letouzé, R., F. Daguin, 1983. Manifestation spontanée et aléatoire d'une croissance anormale en culture *in vitro*. Recherche de marqueurs métaboliques. Rev. Can. Biol. Exp., 42, 23-28.

Letouzé, R., F. Daguin, 1987. Control of vitrification and hypolignification process in *Salix babylonica* cultured *in vitro*. Acta Hortic., 212, 185-191.

Liners, F., Th. Gaspar, P. Van Cutsem, 1994. Acetyl- and methyl-esterification of pectins of friable and compact sugar-beet calli: consequences for intercellular adhesion. Planta, 192, 545-556.

Lovatt, C. J., 1990. Stress alters ammonia and arginine metabolism. In: Polyamines and Ethylene: Biochemistry, Physiology and Interactions. Eds. H. E. Flores, R. N. Arteca, J. C. Shannon, American Society of Plant Physiology, Rockville MD, 166-179.

Mahmound, G. S., T.B. Melo, 1991. The effect of the antioxidant spermine on the photophysical processes and photodegradation of tryptophan in aqueous solution at 293 K.J. Photochem. Photobiol., 9, 355-367.

Memelink, J., J.H.C. Hoge, R.A. Schilperoort, 1987. Cytokinin stress changes the development regulation of several defence-related genes in tobacco. The EMBO J., 6, 3579-3583.

Mérillon, J.M., M. Filali, P. P. Dupéron, M. Montagu, J.C. Chénieux, M. Rideau, 1995a. Effect of 2,4-dichlorophenoxyacetic acid and habituation on lipid and protein composition of microsomal membranes from periwinkle cell suspensions. Plant Physiol. Biochem., 33, 443-451.

Mérillon, J.M., F. Huguet, B. Fauconneau, M. Rideau, 1995b. Specific binding of verapamil to microsomal membranes of *Catharanthus roseus* cell suspensions. J. Plant Physiol., 146, 279-282.

Mérillon, J.M., Montagu, J.C. Chénieux, M. Rideau, 1994. Modification des lipides membranaires et réponse aux cytokinines de cellules de *Catharanthus roseus* cultivées in vitro. Bull. Soc. Pharm. Bordeaux, 133, 141-152.

Mérillon, J.M., L. Ouelhazi, P. Doireau, J.C. Chénieux, M. Rideau, 1989. Metabolic changes and alkaloid production in habituated and non-habituated cells of *Catharanthus roseus* grown in hormone-free medium. Comparing hormone-deprived non-habituated cells with habituated cells. J. Plant Physiol., 134, 54-60.

Mérillon, J.M., K. Ramawat, F. Andreu, J.C. Chénieux, M. Rideau, 1986. Accumulation alcaloïdique dans des lignées cellulaires de *Catharanthus roseus* entretenues en présence ou en absence de phytohormones. C.R. Acad. Sci. Paris, 303, III, 689-692.

Oberley, L.W., T.D. Oberley, 1988. Role of antioxidant enzymes in cell immortalization and transformation. Molec. Cell. Biochem., 84, 147-153.

Ouelhazi, L., M. Filali, A. Décendit, J.C. Chénieux, M. Rideau, 1993. Differential protein accumulation in zeatin- and 2,4-D-treated cells of *Catharanthus roseus*. Correlation with indole alkaloid biosynthesis. Plant Physiol. Biochem., 31, 421-431.

Pâques, M., P. Boxus, 1987. Vitrification: review of literature. Acta Hortic., 212, 155-166.

Phan, C.T., 1991. Vitreous state *in vitro* culture: ethylene versus cytokinin. Plant Cell Rep., 9, 517-519.

Phan, C.T., R. Letouzé, 1983. A comparative study of chlorophyll, phenolic and protein content and of hydroxycinnamate: CoA ligase activity of normal and vitreous plants (*Prunus avium* L.) obtained *in vitro*. Plant Sci. Lett., 31, 323-327.

Sankhla, D., 1994. Toxic oxygen accumulation and hyperhydricity. In: Agricell Report. Eds E.B. Herman, Agritech Consultant, Inc, vol. 23, number 2.

Slocum, R. D., L.H. Weinstein, 1990. Stress-induced putrescine accumulation as a mechanism of ammonia detoxification in cereal leaves. In: Polyamines and Ethylene: Biochemistry, Physiology and Interactions. Eds. H. E. Flores, R. N. Arteca, J. C. Shannon, Rockville MD, American Society of Plant Physiology, 157-165.

Smigocki, A.C., L.D. Owens, 1988. Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. Proc. Natl. Acad. Sci. USA, 85, 5131-5135.

Srivastava, H., R.P. Singh, 1987. Role and regulation of L-glutamate dehydrogenase activity in higher plants. Phytochemistry, 26, 597-610.

Stonier, T., 1970. The role of auxin protectors in autonomous growth. In: Les Cultures de Tissus de Plantes. Eds. Hirth, G. Morel, coll. Intl. CNRS, Strasbourg, 423-435.

Stonier, T., H.M. Yang, 1971. Studies on auxin protectors. X. Protector levels and lignification in sunflower crown gall tissue. Physiol. Plant., 25, 474-481.

Stonier, T., H.M. Yang, 1973. Studies on auxin protectors. XI. Inhibition of peroxidase-catalyzed oxidation of glutathione by auxin protectors and β-dihydroxyphenols. Plant Physiol., 51, 391-399.

Sutter, E.G., R.W. Langhans, 1979. Epicutilar wax formation on carnation plantlets regenerated from shoot tip culture. J. Am. Soc. Hortic. Sci., 104, 493-496.

Thomas, J.C., E.F. McElwain, H.J. Bohnert, 1992. Convergent induction of osmotic stress-responses, abscisic acid, cytokinin and effects of NaCl. Plant Physiol., 100, 416-423.

Thompson, J.E., R.L. Legge, R.F. Barber, 1987. The role of free radicals in senescence and wounding. New Phytol., 105, 317-344.

Vianello, A., F. Macri, 1991. Generation of superoxide anion and hydrogen peroxide at the surface of plant cells. J. Bioenerg. Biomembr., 23, 409-423.

Vieitez, A.M., A. Ballester, M.C. San-Jose, E. Vieitez,. 1987. Vitrification in chesnut shoots regenerated *in vitro*. Acta Hortic., 212, 231-234.

Williams, R.R., A.M. Taji, 1991. Effect of temperature, gel concentration and cytokinins on vitrification of *Olearia microdisca* (J.M. Black) *in vitro* shoot cultures. Plant Cell Tissue Organ Cult., 26, 1-6.

Winston, G.W., 1990. Physicochemical basis for free radical formation in cells: production and defenses. In: Stress Responses in Plants: Adaptation and Acclimation Mechanisms. Eds R.G. Alscher, J.R. Cumming, Wiley-Liss Inc., New-York, 57-86.

Yamaha, T., A. Oak, 1987. Synthesis of glutamate by mitochondria: an anaplerotic function for glutamate dehydrogenase. Physiol. Plant., 70, 749-756.

Zimmerman, T.W., S.M.D. Rogers, B.G. Cobb, 1991. Controling vitrification of petunia *in vitro*. In vitro Cell Dev. Biol., 27, 165-167.

Ziv, M., 1991. Vitrification: morphological and physiological disorders of *in vitro* plants. In: Micropropagation Technology and Applications. Eds. P.C. Debergh , R.H. Zimmerman, Kluwer Acad. Publ., Dordrecht, 45-69.

Ziv, M., G. Meir, A.H. Halevy, 1983. Factors influencing the production of hardened glaucus carnation plantlets *in vitro*. Plant Cell Tissue Organ Cult., 2, 55-65.

Ziv, M., A. Schwartz, D. Fleminger, 1987. Malfunctionning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implications for hardening. Plant. Sci., 52, 127-134.